

### REMARKS

The presently claimed invention features methods for identifying candidate therapeutic agents for the treatment of prostate cancer. The claimed methods entail screening test compounds to identify those compounds that reduce the expression of alpha-methylacyl CoA racemase ("racemase") mRNA.

Claim 34 has been amended. Claims 59-79 have been added. Support for claims 59-65 is found in the specification, for example on page 22. Support for claims 66-72 is found in the specification, for example on page 23. Support for claims 73-75 is found in the specification, for example on page 49. Support for claims 59-65 is found in the specification, for example on page 26. No new matter has been added. Moreover, the newly added claims depend from the previously pending which have already been searched and examined.

### Rejections Under 35 U.S.C. §112, second paragraph

The Examiner rejected claim 34 as allegedly indefinite in reciting the phrase "selectively hybridizes". The Examiner argued that the term selectively hybridizes is not defined by the claim and thus "reads on the full range of selectivity, that is from very to very high selectivity". According to the Examiner, "one of ordinary skill in the would not be reasonably apprised of the scope of the invention and would not be able to determine the metes and bounds of the claims".

Applicants have deleted the term "selectively" from claim 34. Applicants have so solely to address the Examiner's rejection. However, Applicants maintain that the term is well understood by those of ordinary skill in the art.

Claim 34 now refers to a nucleic acid molecule that hybridizes to racemase mRNA under specified stringent hybridization and washing conditions. Thus, the hybridizing nucleic acid molecule is described in definite and concrete terms that are readily understood by those of ordinary skill in the art. Indeed, identifying a nucleic acid molecule as one that hybridize to a particular nucleic acid molecule under defined stringent hybridization and washing conditions is

a routine matter for those of ordinary skill in the art. Thus, one of ordinary skill in the art can readily determine the metes and bounds of claim 34.

Rejections Under 35 U.S.C. §112, first paragraph

The Examiner rejected claims 33 and 34 under 35 U.S.C. §112, first paragraph as allegedly not enabled. Applicants respectfully traverse this rejection.

The Examiner stated that the specification does not enable one skilled in the art to use the claimed methods to identify candidate therapeutic agents for the treatment of prostate cancer. The Examiner argued that the claims are not enabled because "it is unpredictable that any of the screened compounds would have therapeutic effect for the treatment of prostate cancer in view of the teaching in the art that anticancer drug discovery for cancer therapy treatment is unpredictable ... and further in view that gene therapy and *in vivo* therapy using antisense is unpredictable." The Examiner noted the teachings of Beach et al., Kuefer et al., Luo et al., Rubin et al., and Jiang et al., discussed by Applicants in the prior amendment. However, the Examiner argued that these teachings are not persuasive because they do not teach "that prostate cancer could be treated by a compound that decreases the level of mRNA expression of the raceemase". Applicants disagree with the Examiner's conclusions.

First, the Examiner is not applying the correct legal standard for enablement. It appears that the Examiner would find the claims to be enabled only if it could be definitively demonstrated that compounds that decrease racemase mRNA expression are efficacious for treating prostate cancer. The enablement standard does not require that it be conclusively demonstrated that compounds that decrease racemase mRNA expression are efficacious for treating prostate cancer. Rather, the claims are enabled if one skilled in the art, based on the specification, would know how to use the claimed methods to identify inhibitors of racemase mRNA expression that are candidate therapeutic agents. The claimed methods are enabled if they allow one skilled in the art screen a large number of compounds in order to identify a subset that are candidate therapeutic agents for the treatment of prostate cancer. Applicants submit that each and every step of the claimed methods are in fact in the specification. For example,

screening methods for identifying compounds that alter racemase expression and sources of test compounds are described on pages 39-46 of the specification. Useful probes for measuring racemase mRNA expression are taught on pages 22-24 and 26-28 of the specification. Useful antibodies for measuring racemase protein expression are taught on pages 31-33 of the specification. Thus, the claimed methods are fully enabled.

Second, even if racemase does not cause prostate cancer, it is clearly an indicator of prostate cancer. Where a compound decreases expression (directly or indirectly) of an indicator of prostate cancer, one skilled in the art would view the compound as a potential therapeutic agent because reduced expression of the indicator of prostate cancer may indicate a reduction in prostate cancer.

Finally, as detailed below, it is evident that there is a sound scientific basis for concluding that inhibitors of racemase mRNA expression could be useful as therapeutic agents for the treatment of prostate cancer. This conclusion is not dependent on determining that increased racemase mRNA expression definitely causes prostate cancer, as the examiner appears to suggest.

#### **Inhibition of racemase mRNA reduces the proliferation of prostate cancer cells**

Zha et al. (*Cancer Res.* 63:7365, 2003; Exhibit A) report that inhibition of racemase mRNA by small interference RNA (siRNA) reduces the expression of racemase enzyme and impairs proliferation of androgen-responsive LAPC-4 cells, a prostate cancer cell line. Zha et al. also report that LAPC-4 cells treated with siRNA specific for racemase undergo cell cycle arrest at G(2)-M. Finally, Zha et al. report that simultaneous inhibition of racemase mRNA by siRNA and androgen signaling by androgen withdrawal suppresses the growth of LAPC-4 cells to a greater extent than either treatment alone. Based on these studies, Zha et al. conclude that racemase "is essential for optimal growth of [prostate cancer] cells *in vitro*" and that racemase "has the potential to be a complementary target with androgen ablation in [prostate cancer] treatment."

**Increased racemase mRNA and protein expression is observed in clinical prostate cancer samples**

As described in the present specification, Applicants found that racemase mRNA and protein is expressed at a higher level in actual clinical prostate tumor sample and prostate tumor metastases than in clinical samples of normal prostate tissue. As discussed previously, numerous studies on large patient populations have confirmed this result (see, e.g., Beach et al. 2002 *Am. J. Surgical Pathol.* 26:1588; Kuefer et al. 2002 *Am. J. of Pathol.* 161:841; Luo et al. 2002 *Cancer Res.* 62:2220; Rubin et al. 2002 *JAMA* 287:1662; and Jiang et al. 2001 *Am. J. Surgical Pathol.* 25:1397).

**The biological activity of racemase is consistent with a role in prostate cancer**

As discussed previously, Luo et al. (*Cancer Res.* 62:2220, 2002) explain that certain aspects of the biological activity of racemase may have particular relevance for prostate cancer. Racemase catalyzes the conversion of (R)-I-methyl branched-chain fatty acyl-CoA esters to (S)-stereoisomers, which, unlike the (R)-stereoisomers, can serve as substrates for branched-chain acyl-CoA oxidase during peroxisomal oxidation. Luo et al. explain that two aspects of this oxidation pathway “may have particular relevance for prostate carcinogenesis: (a) the main sources of branched-chain fatty acids in humans have been implicated as dietary risk factors for prostate cancer; and (b) peroxisomal  $\beta$ -oxidation generates hydrogen peroxide, a potential source of pro-carcinogenic oxidative damage (Luo at page 2220, citations omitted).” The results of Luo et al. support the proposition that racemase plays a role in the development and/or progression of prostate cancer.

**Racemase sequence variants are associated with increased prostate cancer risk**

As discussed previously, Zheng et al. (2002 *Cancer Res.* 62:6485) conclude that sequence variants in the racemase gene are associated with prostate cancer risk. This study examined 17 sequence variants, including 10 missense mutations in 159 HPC families, 245 sporadic prostate cancer cases and 222 non-prostate cancer controls. The fact that certain

racemase sequence variants are associated prostate cancer risk further supports the proposition that racemase plays a role in the development and/or progression of prostate cancer.

**The claims are enabled irrespective of the unpredictability of gene therapy or antisense therapy**

The Examiner argued that the claims are not enabled because gene therapy and *in vivo* antisense therapy are “unpredictable”. The Examiner provides a detailed discussion of some of the difficulties that can occur in gene therapy and antisense therapy. However, the Examiner’s concerns are misplaced and undue. Assuming, without conceding, that gene therapy and antisense therapy are not useful for treating prostate cancer and that nucleic acids are not useful as test compounds in the claimed screening methods, there remain many, many compounds, e.g., small molecules that can be screened and may prove to be useful therapeutic agents. Thus, reconsideration and withdrawal of the rejection is requested.

**The use of nucleic acid molecules that hybridize to SEQ ID NO:3 is enabled**

The Examiner rejected claim 34 as allegedly not enabled because, according to the Examiner, “it is not clear whether the nucleic acid molecule that ‘selectively’ hybridizes to SEQ ID NO:3 used in the claimed method would be a probe specific for SEQ ID NO:3”.

As discussed above claim 34 has been amended to delete the term “selectively”. Claim 34 now refers to a nucleic acid molecule that hybridizes to racemase mRNA under specified stringent hybridization and washing conditions. A nucleic acid molecule that hybridizes to racemase mRNA under stringent hybridization conditions is a probe that is sufficiently specific for racemase mRNA to identify racemase mRNA. Those skilled in the art routinely use probes that hybridize under stringent hybridization and washing conditions to a particular nucleic acid molecule to identify and quantify the particular nucleic acid molecule. Thus, the hybridizing nucleic acid molecule in claim 34 is defined in a manner that allows one of ordinary skill and art to use the claimed method.

In rejecting claims 34 as allegedly not enabled, the Examiner stated that "the condition in which the test sample is exposed to the nucleic acid probe is not known". Because of this, according to the Examiner, "one would expect that unrelated sequences would be detected by the claimed method". Applicants see no reason to define within the claim the conditions under which the test sample is exposed to the probe. Those skilled in the art know perfectly well how to detect a particular molecule using a hybridizing probe. This is a routine application of basic molecular biology.

In view of the forgoing, Applicants respectfully request that the rejection under 35 U.S.C. §112, first paragraph be withdrawn.

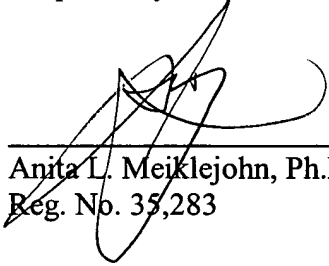
CONCLUSION

It is believed that the claims are in condition for allowance.

Enclosed is a Petition for Extension of Time with the appropriate fee. Please apply any other charges or credits to deposit account 06-1050.

Respectfully submitted,

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# $\alpha$ -Methylacyl-CoA Racemase as an Androgen-Independent Growth Modifier in Prostate Cancer<sup>1</sup>

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## ABSTRACT

$\alpha$ -Methylacyl-CoA racemase (AMACR) is an enzyme involved in  $\beta$ -oxidation of branched-chain fatty acids and bile acid intermediates. Recent work has identified AMACR as a new diagnostic marker for prostate cancer (PCa). The data from the present study suggest that AMACR is also functionally important for the growth of PCa cells. Overexpressed AMACR from both clinical tissues and PCa cell lines is wild type by sequence analysis and functionally active by enzymatic assay. Correspondingly, enzyme activity of AMACR increases ~4-fold in PCa in comparison with adjacent normal prostate. Small interference RNA (siRNA) against AMACR, but not the control inverted siRNA, reduced the expression of AMACR and significantly impaired proliferation of the androgen-responsive PCa cell line LAPC-4. No effect was observed in HeLaS3 cells, which express AMACR at a low level. Cell cycle analyses revealed a G<sub>2</sub>-M cell cycle arrest in LAPC-4 cells treated with siRNA compared with mock treatment or control inverted siRNA. Expression of a siRNA-resistant form of AMACR in LAPC-4 cells protects the cells from growth arrest after AMACR-specific siRNA treatment. Data from Western blotting and luciferase-based reporter assays suggest that the function and expression of AMACR are independent of androgen receptor-mediated signaling. Moreover, simultaneous inhibition of both the AMACR pathway by siRNA and androgen signaling by means of androgen withdrawal or antiandrogen suppressed the growth of LAPC-4 cells to a greater extent than either treatment alone. Taken together, these data suggest that AMACR is essential for optimal growth of PCa cells *in vitro* and that this enzyme has the potential to be a complementary target with androgen ablation in PCa treatment.

## INTRODUCTION

PCa<sup>3</sup> is the most commonly diagnosed noncutaneous neoplasm in Western countries (1). In the United States, it is the second leading cause of cancer death in men following lung cancer (2). Whereas localized disease can be effectively treated, if metastasis occurs, the frequent low proliferation index of PCa presents a challenge for conventional chemotherapies. Androgen ablation via castration and/or administration of small chemical inhibitors (e.g., luteinizing hormone-releasing hormone agonists and AR antagonists) is the most common treatment for advanced PCa. Despite a rapid initial response in the majority of cases, PCa will ultimately progress to a hormone-refractory stage, for which no curative therapies currently exist. Thus, extensive efforts are under way to better understand the initiation and development of the disease in hope of finding better diagnostic markers and therapeutic targets for PCa.

With the development of microarray technology, a number of genes have been identified that are consistently up-regulated in PCa with reference to normal prostate and benign prostatic hyperplasia. These genes have the potential to complement PSA as new and perhaps better diagnostic markers for PCa. One of these genes is AMACR (3–5). In adults, the expression of AMACR is high in liver and kidney, whereas an intermediate level of expression is seen in the intestinal tract. During tumorigenesis in the prostate, AMACR expression is elevated 7–8-fold at both the mRNA and protein levels, as evidenced by real-time PCR analysis and Western blotting. Large-scale tissue microarray studies indicate that AMACR expression starts early in prostate oncogenesis, with ~70% of high-grade prostatic intraepithelial neoplasia staining positive. In primary PCa, >95% of cases are positive for AMACR (6), whereas <1% of adjacent normal prostate epithelial cells from the tumor-containing glands have visible AMACR staining. Once AMACR protein is expressed, its level remains elevated as the PCa progresses to higher grades and stages and even metastasizes (6–8). The consistent and robust expression of AMACR establishes this protein as an important new diagnostic marker for PCa, especially in the case of needle biopsies, when the quantity and quality of tissue are limited (6, 8).

AMACR is a peroxisomal and mitochondrial enzyme capable of racemizing the  $\alpha$ -carbon of various  $\alpha$ -methylacyl-CoA derivatives (9). Specifically, AMACR is responsible for converting the  $\alpha$ -methyl group of C27-bile acyl-CoAs and pristanoyl-CoA from (*R*) to their (*S*) stereoisomers, which are the only stereoisomers that can be degraded via  $\beta$ -oxidation in peroxisomes and mitochondria. Pristanic acid, the precursor of pristanoyl-CoA, is derived from dietary sources either directly or through  $\alpha$ -oxidation from phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), which is also derived exclusively from the diet in humans. The role that localized, up-regulated expression of AMACR plays in prostate carcinogenesis, if any, is undefined. Systemic loss of AMACR activity has been observed and shown to have phenotypic consequences. Ferdinandusse *et al.* (10) recently described patients with sensory motor neuropathy associated with homozygous loss of function germ-line mutations of AMACR. Others reported a neonatal cholestasis case (11) and a vitamin K deficiency resulting from malabsorption related to bile acid dysfunction (12) secondary to AMACR mutations. In all these cases, dietary modification and/or bile acid supplementation offer an effective treatment for the symptoms of AMACR deficiency. This condition is rare, and none of the patients have been studied for incidence of PCa in particular.

To further characterize the dysregulation of AMACR that accompanies prostate carcinogenesis and to begin to assess a possible functional role in this process, we analyzed the enzymatic activity of AMACR in both clinical samples of PCa and tissue culture cells, and then we asked whether AMACR is essential for the proliferation of PCa cell lines. We found that decreasing AMACR protein levels in PCa cells leads to decreased proliferation and that this effect appears to be completely independent of androgen action. We then assessed the therapeutic potential of down-regulating AMACR in combination with androgen ablation *in vitro*, and we found an additive antiprolif-

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<sup>3</sup> The abbreviations used are: PCa, prostate cancer; AMACR,  $\alpha$ -methylacyl-CoA racemase; siRNA, small interference RNA; AR, androgen receptor; PSA, prostate-specific antigen; FBS, fetal bovine serum; CS, charcoal-stripped; CMV, cytomegalovirus; PSE, PSA enhancer; PB, probasin promoter; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; UTR, untranslated region.

erative effect. Our results indicate that further study of AMACR as a novel therapeutic target in PCa is warranted.

## MATERIALS AND METHODS

**Tissues, Cell Lines, Chemicals, Plasmids, and Antibodies.** For Western blotting and enzymatic activity analysis and immunofluorescence, fresh tissues were harvested as described previously from patients undergoing prostatectomy at The Johns Hopkins Hospital for the treatment of PCa (13). The human PCa cell lines LNCaP, DU145, PC-3, and CWR22-Rv1 were acquired from American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640/10% FBS (Life Technologies, Inc., Grand Island, NY) at 37°C with 5% CO<sub>2</sub>. The human PCa cell line LAPC-4 (14) was obtained from Dr. John Isaacs at Johns Hopkins University and maintained in Iscove's modified DMEM/10% FBS and 1 nM R1881 (Life Technologies, Inc.) at 37°C with 5% CO<sub>2</sub>.

The synthetic androgen methyltrienolone (R1881) was purchased from New England Nuclear (Boston, MA). Casodex (bicalutamide) was a gift from AstraZeneca (London, United Kingdom). For all R1881 treatment procedures, cells were incubated in media containing 10% CS-FBS (Cocalico Biologicals, Reamstown, PA) for at least 24 h before the addition of the same media (fresh) including R1881.

Control plasmids pGL3<sub>Basic</sub> (pGL3) and pRL-TK were obtained from Promega (Madison, WI) and used for both luciferase assay optimization and normalization. Empty pcDNA 3.1(+/-) was purchased from Invitrogen (Carlsbad, CA) and used to compensate for unequal DNA/CMV promoter levels in transfection/luciferase assays. The wild-type AMACR cDNA was PCR amplified from LNCaP PCa cells using primers with *Bgl*II and *Sal*I linkers at each end, sequenced, and inserted into pcDNA3.1(-). Loss of function mutation (L107P; Ref. 10) and the siRNA-resistant form of AMACR were generated by site-specific mutagenesis (15). AR activity was measured with the luciferase reporter construct pBK-PSE-PB (provided by Dr. Ron Rodriguez; Johns Hopkins University). pBK-PSE-PB consists of the human PSE [1592-bp *Bam*HI/*Bgl*II fragment from the CN-65 construct; see Schuur *et al.* (16)] fused to the rat PB [-421 to +42 of sequence reported by Rennie *et al.* (17)]. This reporter permits efficient AR-driven activity because it contains four androgen response elements, two in the PSE and two in the PB. pcDNA3.1(-)hAR was generated as described previously and used for expression of wild-type human AR (18). The AMACR reporter (pGL3<sub>Basic</sub>-AMACR) contains -1711 to +86 bp of the AMACR 5'-proximal region including the full-length 5'-UTR. Using genomic DNA, PCR with *Pfu* polymerase (Stratagene, La Jolla, CA) was performed to amplify the appropriate fragment, which was subsequently cloned using the pTOPO Blunt kit (Invitrogen). The promoter was then subcloned through restriction sites (*Xho*I from pTOPO and *Nco*I from the cloned sequence) into pGL3<sub>Basic</sub>.

Anti-AMACR rabbit polyclonal antibody was generated as described previously (9). The other antibodies were obtained from PharMingen (San Diego, CA; mouse anti-AR monoclonal antibody), Calbiochem (La Jolla, CA; anti- $\alpha$ -tubulin), DAKO (Glostrup, Denmark; rabbit polyclonal antibody antihuman PSA), the Binding Site (Birmingham, United Kingdom; sheep antihuman catalase for immunocytochemistry), Sigma (rabbit antihuman catalase for Western blotting), Jackson ImmunoResearch Laboratories (West Grove, PA; rhodamine-conjugated donkey antisheep IgG), Molecular Probes (Eugene, OR; Alexa Fluor 488-conjugated donkey antirabbit IgG, mouse anti-cytochrome *c* oxidase subunit I monoclonal antibody), and Pierce (Rockford, IL; horseradish peroxidase-conjugated goat antimouse and antirabbit IgG).

**Immunocytochemistry.** Immunocytochemistry was performed essentially as reported previously (13). Briefly, paraffin slides were deparaffinized, steamed (antigen retrieval), blocked (PBS/5% normal donkey serum), and incubated for 1 h at 37°C with primary antibodies diluted at 1:100 (AMACR), 1:50 (catalase), and 1:50 (cytochrome *c* oxidase). As appropriate, slides were then treated with 4,6-diamidino-2-phenylindole dihydrochloride (100 ng/ml; Molecular Probes), rhodamine-conjugated antimouse or sheep IgG (1:400), and/or Alexa Fluor 488-conjugated antirabbit IgG (1:2000); washed; mounted with Prolong Antifade (Molecular Probes); and viewed under a fluorescence microscope (AxioScope; Carl Zeiss, Thornwood, NY). Images were documented using IP-Lab image-capturing software. Nonspecific background was monitored by treating specimens with secondary antibody only. In all cases, adjacent slides were also standard stained with H&E afterward for histological documentation.

**Western Blot Analysis.** Cell lysates were prepared by first washing cells once with phosphate buffer and then extracting with lysis buffer [4% SDS, 100 mM Tris-HCl (pH 7.4), and 1 mM EDTA]. Protein concentrations were determined using a bicinchoninic acid assay (BCA; Pierce). Techniques for running SDS-PAGE and Western blots were as described in Molecular Cloning (J. Sambrook and D. W. Russell, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Prestained molecular weight standards were from Bio-Rad (Hercules, CA). On completion of electrophoresis, proteins were transferred for Western blot to nitrocellulose (Bio-Rad) and probed in 5% nonfat dried milk/PBS/0.2% Tween 20. After washing, the membranes were probed with horseradish peroxidase-conjugated secondary antibody (anti-IgG). Enhanced chemiluminescence was performed with the ECL kit (Amersham-Pharmacia, Piscataway, NJ) followed by autoradiography. For appropriate blots,  $\alpha$ -tubulin levels were also determined to verify equal loading.

**siRNA Synthesis and Transfection.** Chemical synthesized sense and antisense RNAs corresponding to AMACR cDNA sequence (AAG-GCA-GAG-UGG-UGU-CAA-AUC, nucleotides 903-923 from NM\_014324, NCBI) and the inverted control RNA (CUA-AAC-UGU-GGU-GAG-ACG-GAA) were purchased from Xeragon Inc. (Huntsville, AL). The second set of siRNA nucleotides targeting AMACR (siRNA<sub>2</sub>, AAG-AAG-AAG-UUU-GCA-GAU-GUA, nucleotides 867-887 from NM\_014324) was purchased from Dharmacon (Lafayette, CO). For siRNA transfection, cells at exponential growth were harvested by trypsinization. The trypsin was neutralized with 1:1 (v/v) medium/10% FBS. The pellet was washed once with appropriate culture medium (without FBS) and resuspended at  $1.25 \times 10^7$  cells/ml. Cells (0.4 ml;  $\sim 5 \times 10^6$ ) were mixed with 20  $\mu$ l of 20  $\mu$ M siRNA. Electroporation was performed in a 0.4-cm cuvette (Bio-Rad) at 250 V and 960  $\mu$ F in a Bio-Rad Gene Pulser Electroporator. For the luciferase assay, the cells were replated into appropriate 96-well plates as described below, followed by androgen depletion, transfection, and luciferase assay. For the proliferation assay, the cells were recovered on a 60-mm dish with 2.5 ml of growth medium containing 10% FBS (and also containing 1 nM R1881 for LAPC-4 cells). Two days later, the electroporations were repeated to maintain AMACR suppression long enough for the proliferation assay. The cells were immediately plated with serum-containing medium into 96-well plates for the proliferation assay ( $\sim 2500$  viable cells/well for LAPC-4; Cell Titer96 Aqueous One Solution Cell Proliferation Assay from Promega), on 24-well plates for Western blotting or cell cycle analysis ( $\sim 1 \times 10^5$  cells/well), and on 24-well plates for colony formation assay ( $\sim 250$  viable cells/well).

**Transfection and Luciferase Assays.** Luciferase assay experiments were performed essentially as described previously (18). Briefly, cells were plated at medium density to clear-bottomed, opaque-walled, 96-well plates (Isoplate TC; Perkin-Elmer Wallac, Gaithersburg, MD). With the experiments involving androgen treatment, cells were given media/10% CS-FBS 24 h after plating (day 2), transfected (day 3), treated with R1881 (day 4), and analyzed for luciferase activity (day 5). Transfections were performed using FuGENE 6 (Roche) at 1  $\mu$ l/0.2  $\mu$ g DNA for CWR22-Rv1 cells or LipofectAMINE 2000 (Invitrogen) at 0.25  $\mu$ l/96-well plate well for LAPC-4 cells. To compensate for unequal DNA/CMV promoter levels between certain transfection groups, pcDNA was included as appropriate. pRL-TK (10 ng), which encodes *Renilla* luciferase, was included in all transfections to normalize the transfection efficiency. Luciferase assays were performed as described using nonproprietary substrate/buffer mixes (19). Cells were washed once in PBS and lysed in 30  $\mu$ l of 1 $\times$  Passive Lysis Buffer (Promega). Using the Wallac 1450 Microbeta Jet luminescence reader, firefly and *Renilla* luciferase substrate mixes (100  $\mu$ l) were injected sequentially, allowing a 10-second readout for each enzyme. All experiments were repeated at least three times, and all experimental groups therein were performed in replicates of six. To control for transfection efficiency for individual wells, firefly luciferase values were normalized to the corresponding *Renilla* values.

Stable clones expressing the siRNA-resistant form of AMACR were generated by transfecting 4  $\mu$ g of pcDNA3.1-AMACR-siR into 80% confluent LAPC-4 cells in a 6-well plate by LipofectAMINE 2000 (Invitrogen). The next day, one-tenth of the cells were plated into a 100-mm plate and selected for 14 days with 400 ng/ml G418 (Invitrogen). Pooled clones were used in siRNA treatment and proliferation assay as described above.

**Flow Cytometry, Proliferation Assay, and Colony Formation Assay.** The cell cycle distribution was determined by flow cytometry measuring DNA content by propidium iodide staining (20). Briefly, the cells were washed,



fixed, digested with trypsin (0.03 mg/ml in PBS) for 10 min, incubated with RNase A (0.1 mg/ml in PBS) for 10 min at room temperature, and then stained with propidium iodide (0.4 mg/ml). Propidium iodide and forward light scattering were detected by using a Coulter EPICS 752 flow cytometer equipped with MDADS 11 software, Version 1.0.

Cell proliferation assays were performed with the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), which uses a tetrazolium compound (MTS) as substrate. The number of viable cells is proportional to the level of formazan-like compound produced from reduction of MTS by NAD(P)H. LAPC-4 cells (2500 cells) or HeLaS3 cells (500 cells) were plated in clear 96-well plates (Falcon) 1 day after the second electroporation. For combination with androgen withdrawal, media were replaced 24 h later with media containing 10% CS-FCS with or without 1 nM R1881. Measurements of relative cell number were taken by adding MTS reagent to wells, incubating cells for 1 h at 37°C, and reading absorbance at 490 nm. Day 0 measurements were taken immediately after R1881 addition/withdrawal, and all measurements thereafter were taken at 24-h intervals (one plate for each day).

For the colony formation assay, LAPC-4 (250 viable cells by trypan blue exclusion) were plated into 24-well plates (Falcon) in quadruplicate. For androgen withdrawal, the medium was changed the next day. The cells were incubated for 14–20 days with a medium change every 3 days. On the last day, the cells were fixed with 10% formaldehyde in PBS and stained with crystal violet (0.25% in H<sub>2</sub>O) for 10 min at room temperature and carefully washed for at least five times with H<sub>2</sub>O.

## RESULTS

**Increased Enzymatic Activity of AMACR in PCa.** Previous studies demonstrated that AMACR protein and mRNA are up-regulated in clinical PCa compared with normal prostate; however, no inference on the enzymatic activity of AMACR or the subcellular localization in PCa has been drawn. As a member of the branched-chain fatty acid  $\beta$ -oxidation pathway, proper subcellular localization to mitochondrion and peroxisome is critical for the function of AMACR (9, 21). Previously, immunohistochemical staining of AMACR in PCa elucidated a punctate cytoplasmic pattern (6), consistent with the predicted subcellular localization in peroxisomes and mitochondria. To verify this, immunofluorescent staining of AMACR was performed simultaneously with either the peroxisomal marker, catalase, or the mitochondrial marker, cytochrome *c* oxidase subunit I. Antibodies against catalase or cytochrome *c* oxidase subunit I highlighted both normal prostate and PCa with similar intensity, as seen in Fig. 1A. In contrast, the antibody against AMACR only stained the PCa glands. Colocalization of AMACR with catalase or cytochrome *c* oxidase subunit I at the luminal side of the PCa cells was demonstrated by overlaying the two color channels (Fig. 1A). Based on these data, we conclude that when overexpressed in PCa, AMACR localizes in the functional subcellular compartments predicted from previous studies. The overexpression of AMACR is relatively specific, in that it does not reflect a general overproliferation of either peroxisomes or mitochondria (*i.e.*, because the number of these organelles does not change, and yet the amount of AMACR increases, the concentration of AMACR increases within these organelles).

The cDNAs of AMACR from a series of paired normal and tumor prostate tissues as well as several human PCa cell lines were sequenced. No sequence variants were identified except for the polymorphisms reported previously (Refs. 10 and 22; data not shown). Each sample analyzed, with exception of the cell line PC-3, was heterozygous for the AMACR gene, as evidenced by two different haplotypes present in the cDNA sequences. These results indicate that the AMACR gene that is overexpressed in PCa is typically wild type, at least at this level of analysis.

To examine the functional role of AMACR, we measured its specific enzyme activity in frozen tissues from PCa and normal prostate using (25S)-trihydroxycholestanoyl-CoA as the substrate

(10). Among the four tumor samples analyzed, two (PCa1 and PCa2) were from discrete tumor loci on the same patient, and both have increased AMACR activity, but at different magnitudes (Fig. 1B). A third tumor sample (PCa3) has 10-fold higher AMACR activity when compared with the normal adjacent prostate (N3) from the same gland. Of the "normal" samples analyzed, one sample (N2) had relatively high AMACR protein and activity, even though no obvious cancer foci were found by examining H&E-stained sections immediately before and after the tissue used in enzymatic assay. However, given the multifocal nature of PCa and the fact that this sample is derived from a tumor-containing gland, we could not rule out the possibility that the sections used in the enzymatic assay might include a small cancer lesion, which was also suggested by the corresponding elevation of AMACR protein level (Fig. 1D). Even factoring in the data from this latter sample with the normal samples, the cancer samples on average showed more than 4-fold higher AMACR activity compared with the normal tissue (Fig. 1B). Consistent with the immunofluorescent staining described above in these same samples, no significant difference in catalase activity between normal tissue and tumor was observed (Fig. 1C). Again, the results support the specific dysregulation of AMACR and potentially branched-chain fatty acid  $\beta$ -oxidation but not general peroxisome proliferation in PCa. Furthermore, these results provide the first indication that in addition to protein expression, concomitant AMACR enzymatic activity consistently increases in PCa.

**Expression and Enzyme Activity of AMACR Is Increased in PCa Cell Lines.** Tissue culture cell lines offer convenient models to assess the functional significance of AMACR in PCa. However, changes in metabolic enzymes can be quite sensitive to *in vitro* culture conditions, and adaptation to tissue culture can result in altered enzymatic profiles compared with those occurring *in vivo* (23). To evaluate this, the expression and activity of AMACR were measured in commonly used PCa cell lines (LNCaP, LAPC-4, DU145, PC-3, and CWR22-Rv1) and a sampling of other cancer cell lines by Western blotting. Among these cell lines, the androgen-sensitive PCa cell lines (LAPC-4 and LNCaP) have the highest AMACR protein expression (Fig. 2A). Cell lines derived from liver or kidney (*e.g.*, HepG2 and HEK293) also express significant amounts of AMACR protein, but in other cell lines, expression is minimal (Fig. 2A). As described above, cDNA sequence analysis confirms that all PCa cell lines express wild-type AMACR. The specific activity of AMACR is correlated with its protein level, with LAPC-4 and LNCaP at the high end and DU145 at the low end (Fig. 2B). These data suggest that PCa cell lines, especially LAPC-4, LNCaP, and possibly CWR22-Rv1, retain features of clinical PCa with respect to AMACR. Consistent with the previous results from prostate tissues, the expression of catalase in these cell lines is roughly equivalent across all samples analyzed by Western blotting (Fig. 2A). These data suggest that these cell lines are relevant models for the functional study of AMACR in PCa.

**AMACR Is Required for the Proliferation of PCa Cells *in Vitro*.** To begin to address the functional importance of AMACR in PCa cells, we used siRNA to decrease the expression of AMACR (24). We chose LAPC-4 because it has the highest AMACR protein and enzyme activity among all human PCa cell lines tested. A 21-nucleotide double-stranded RNA corresponding to human AMACR was introduced into LAPC-4 cells by electroporation. As a control, the inverted sequence was also introduced under identical conditions. A significant reduction of AMACR protein level was observed 48 h after the first electroporation, as well as 24 h after the second electroporation. The decrease continued for at least 4 days (Fig. 3A). Mock electroporation or the use of the inverted siRNA (*inv-siRNA*) did not affect the level

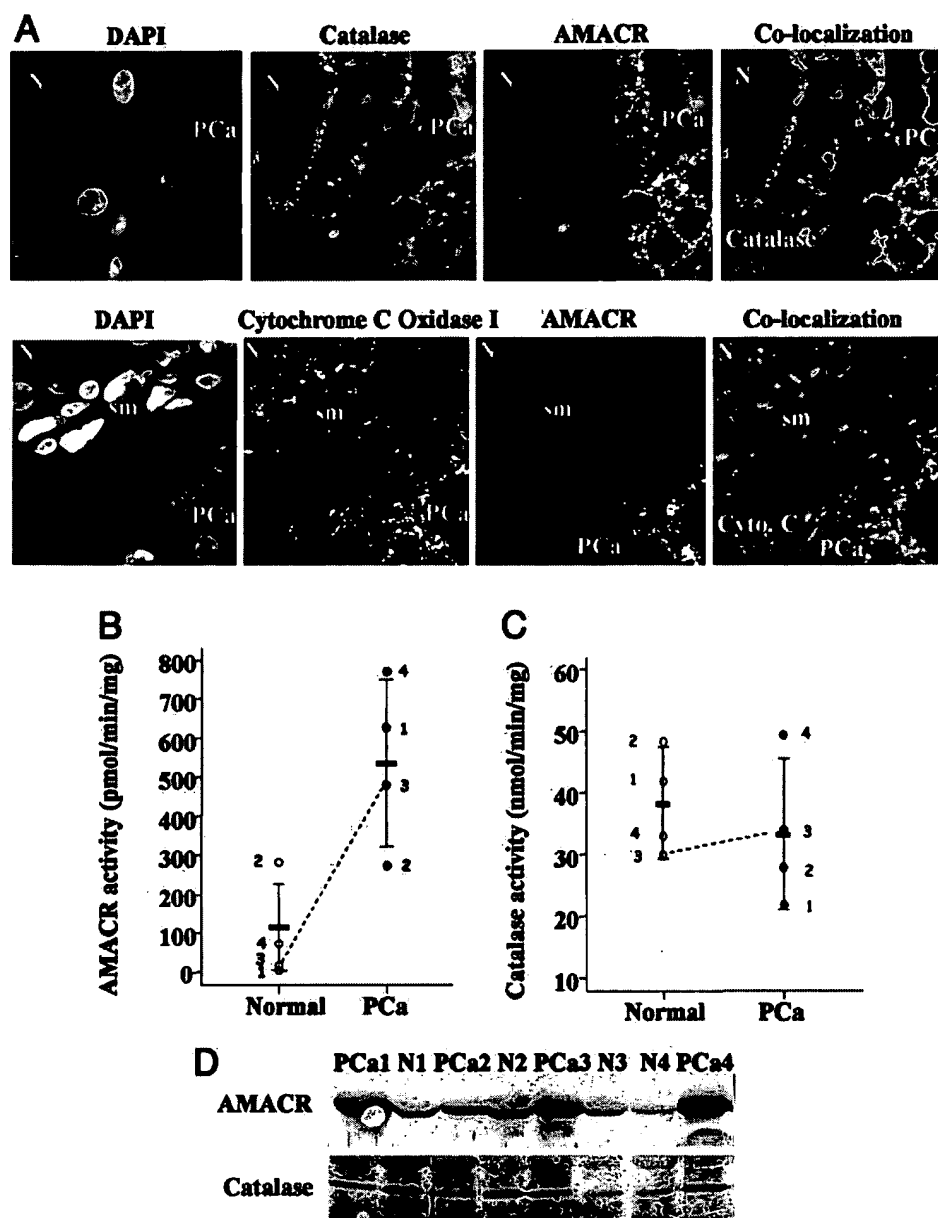


Fig. 1. Expression and enzymatic activity of AMACR in clinical PCa. **A**, immunofluorescence of AMACR with catalase or cytochrome *c* in prostate tissue. Last column (far right) shows the colocalization of AMACR with both mitochondrial and peroxisome markers. **B** and **C**, the activity of AMACR (**B**) and catalase (**C**) was measured as described previously (10) in frozen human prostate tissues (four PCa samples and four adjacent normal prostate samples from tumor-containing prostates). The circles (○, normal; ●, PCa) represent the actual value for each sample; the thin black vertical bars at the top and bottom reflect the SD for each group (PCa or normal). The thick black bars in the middle indicate the average activity for PCa and normal, respectively. The small numbers beside each circle are the sample numbers. Samples N3 and PCa3 were linked with dashed lines to indicate that they were from the same patient. **D**, the Western blot shows the protein level of AMACR and catalase in the same samples used in the activity assay. The sample numbers were also marked correspondingly.

of AMACR during the period of study, consistent with the specificity of the siRNA approach.

The consequences of decreased AMACR expression on cell growth were evaluated. siRNA target AMACR, but not the inverted siRNA, suppressed the proliferation of LAPC-4 cells to 50% of the control value at the third day, and more extensively by day 5 (Fig. 3B). Cell cycle analysis revealed a G<sub>2</sub>-M-phase cell cycle arrest, as evidenced by the doubling of the G<sub>2</sub>-M-phase fraction 2 days after the second electroporation (Fig. 3, D and E). At the same time point, no evident increase in apoptosis (annexin V staining) or decrease in adhesion (crystal violet staining) was seen (data not shown). To test the prolonged effects of temporary loss of AMACR expression, the same number of LAPC-4 cells were plated at low density immediately after the second introduction of siRNA or inverted siRNA and stained by crystal violet after 14 days of culture. The number of colonies formed in siRNA-treated cells was significantly reduced ( $P < 0.01$  in paired Student's *t* test for both siRNA *versus* mock treatment and siRNA *versus* inverted siRNA; Fig. 3C). This result suggests that decreased

AMACR expression imparted a possible long-term growth disadvantage on LAPC-4, which is rather surprising for a short-term gene knockdown approach such as siRNA. Loss of gene suppression by siRNA in long-term culture has been proposed to be a consequence of cell division and subsequent dilution of siRNA molecules (24). In our case, siRNA against AMACR caused a G<sub>2</sub>-M-phase cell cycle arrest, which would prevent the dilution of siRNA in theory. This might explain the persistent growth inhibition observed in the colony formation assays.

Given that most tissue culture cell lines have detectable AMACR protein, it was of interest to assess the specificity of AMACR-dependent proliferation in a non-PCa cell line. AMACR expression was reduced in HeLaS3 cells using the same siRNA strategy. HeLaS3 was chosen because it has a readily detectable expression level of AMACR, albeit lower than that observed in most PCa cell lines, and its exceptional response to the siRNA target gene knockdown approach has been demonstrated in previous studies (24). Although expression of AMACR in HeLaS3 was barely visible after siRNA

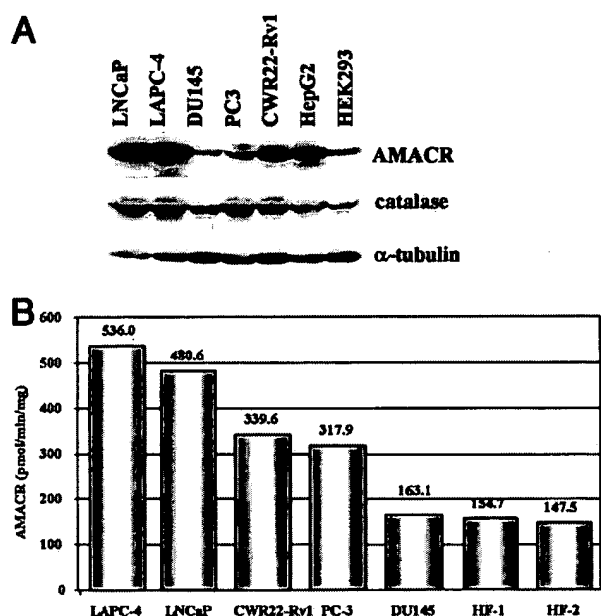


Fig. 2. Expression and activity of AMACR in PCa cell lines. A, the protein level of AMACR, catalase, and  $\alpha$ -tubulin was measured in 50  $\mu$ g of total protein lysates from different cell lines as indicated. B, the activity of AMACR was measured in PCa cell lines using human fibroblasts (HF) as control. The activity value was labeled above each bar.

treatment (Fig. 4A), no evidence of growth inhibition was seen in a 5-day period (Fig. 4B). This result suggests that AMACR is not generally required for cellular proliferation. This is not unexpected, given that cell proliferation defects are not generally noted in AMACR-null patients (10–12).

To further verify that the growth inhibition was indeed caused by loss of AMACR expression, another siRNA sequence targeting a different region of AMACR cDNA (siRNA<sub>b</sub>) was designed. The results using siRNA<sub>b</sub> in both LAPC-4 cells and HeLaS3 cells were consistent with the first siRNA tested (Fig. 4, C–E).

As an additional means to characterize the siRNA-induced growth inhibition, the ability of ectopic expression of AMACR to rescue the cells from growth inhibition was tested. To avoid being degraded by siRNA, two silent mutations in the region targeted by siRNA were introduced into an AMACR expression vector (Fig. 4F). In agreement with the high specificity of siRNA, the expression of this siRNA-resistant form of AMACR did not decrease on introduction of siRNA (Fig. 4G). The decrease of AMACR protein in cells expressing siRNA-resistant AMACR after siRNA treatment is most likely attributable to decreasing the level of endogenous protein. Nevertheless, the resultant protein level was comparable with the endogenous level before expression of the siRNA-resistant AMACR (Fig. 4G). LAPC-4 cells expressing the siRNA-resistant AMACR, but not the neo-resistant control cells (LAPC-4 cells transfected with empty pcDNA3.1 vector), were protected from growth inhibition after siRNA treatment (Fig. 4H). This result supports the loss of AMACR as the reason for the growth inhibition observed. Taken together, these findings also support the conclusion that AMACR is essential for the optimal proliferation of LAPC-4 cells, but not all other cell types.

**Androgen Withdrawal Exaggerates the Growth Inhibition Induced by Loss of AMACR Expression.** Although the LAPC-4 cell line is derived from a hormone-refractory PCa case, it expresses wild-type AR and has androgen-responsive growth characteristics (14, 25). LAPC-4 cells grow maximally in the presence of 1 nM R1881, a synthetic androgen. Withdrawal of androgen from the culture leads to growth inhibition. Increasing androgen (R1881) concentration from 1

to 5 nM did not lead to additional growth advantage or disadvantage in LAPC-4 cells (Fig. 5D), which is different from the biphasic growth response of LNCaP cells (26). These characteristics of LAPC-4 cells provide an *in vitro* model to test AMACR as a potential therapeutic target, especially in combination with androgen ablation therapy.

One day after the siRNA treatment, LAPC-4 cells were cultured in media containing 1 nM R1881, no R1881, or 1 nM R1881 + 10  $\mu$ M Casodex, an antiandrogen. The successful knockdown of AMACR in the presence and absence of R1881 is shown in Fig. 5A. The suppression of AMACR was more complete in the presence of R1881. This is consistently observed in both LAPC-4 and LNCaP (Figs. 5A and 6C). We speculate that androgen deprivation might affect the Dicer-mediated siRNA mechanism. Further study of this observation is under way. When AMACR knockdown was combined with androgen withdrawal or antiandrogen treatment, the proliferation of LAPC-4 cells decreased nearly 90% (Fig. 5B), in contrast to the 50% decrease induced by either treatment alone. When the more long-term effects were measured, very few clones grew out from the doubly treated groups, even after an extended 20-day culture (Figs. 3C and 5C). An additive, if not synergistic, inhibition of growth by loss of both AMACR expression and androgen signaling was observed. Androgen withdrawal alone inhibited the proliferation of LAPC-4 cells without apparent change of cell cycle distribution (Fig. 5F). In contrast, knockdown of AMACR caused the G<sub>2</sub>-M-phase cell cycle arrest with or without androgen (Fig. 5F). This suggests that loss of AMACR expression imposes a different type of growth barrier as compared with androgen deprivation. Consistent with this, increased concentration of synthetic androgen R1881 up to 5 nM could not overcome the growth inhibition resulting from AMACR knockdown (Fig. 5C). The results implicate AMACR as an androgen-independent growth modulator in PCa cells.

#### AMACR Alone Does Not Affect AR-Targeted Gene Expression.

To further study the impact of AMACR on AR action and signaling, the expression of AR itself and an androgen target gene (PSA) was measured under siRNA-treated conditions. Previous studies have shown that although LAPC-4 and CWR22-Rv1 express PSA, the level of endogenous PSA protein does not respond to androgen stimulation consistently (27, 28). However, ligand binding of AR does enhance the protein stability of the receptor in both lines. Thus, this response was used as the surrogate for AR activation. In another PCa cell line, LNCaP, which also expresses AMACR at a high level, PSA expression readily increases on androgen stimulation. By Western blotting, we measured the protein level of AR in LAPC-4 and CWR22-Rv1 cells and PSA in LNCaP cells after siRNA treatment (Fig. 6, A–C). A dramatic increase in AR protein, presumably due to enhanced stability, was evident on exposure to 1 nM R1881 in both LAPC-4 and CWR22-Rv1 (Fig. 6, A and B). siRNA-induced decrease of AMACR expression did not change the steady-state AR protein level in either cell line under both androgen deprivation and androgen stimulation conditions (Fig. 6, A and B). Similar results were obtained for PSA expression in LNCaP cells (Fig. 6C). The results suggest that AMACR expression is not essential for and does not modulate AR signaling.

As an additional method to study the influence of AMACR on AR signaling, luciferase activity derived from a heterologous AR reporter containing a human PSE (16) coupled to the minimal rat PB (Ref. 17; pBK-PSE-PB) was measured as an indicator of AR activation. The AR-positive PCa cell line CWR22-Rv1 was included in this assay because in previous experiments this reporter responded to androgen reproducibly in these cells. LAPC-4 cells were also used, although in this case, ectopic expression of AR from a CMV promoter-driven expression vector was included to counteract the reduction of AR

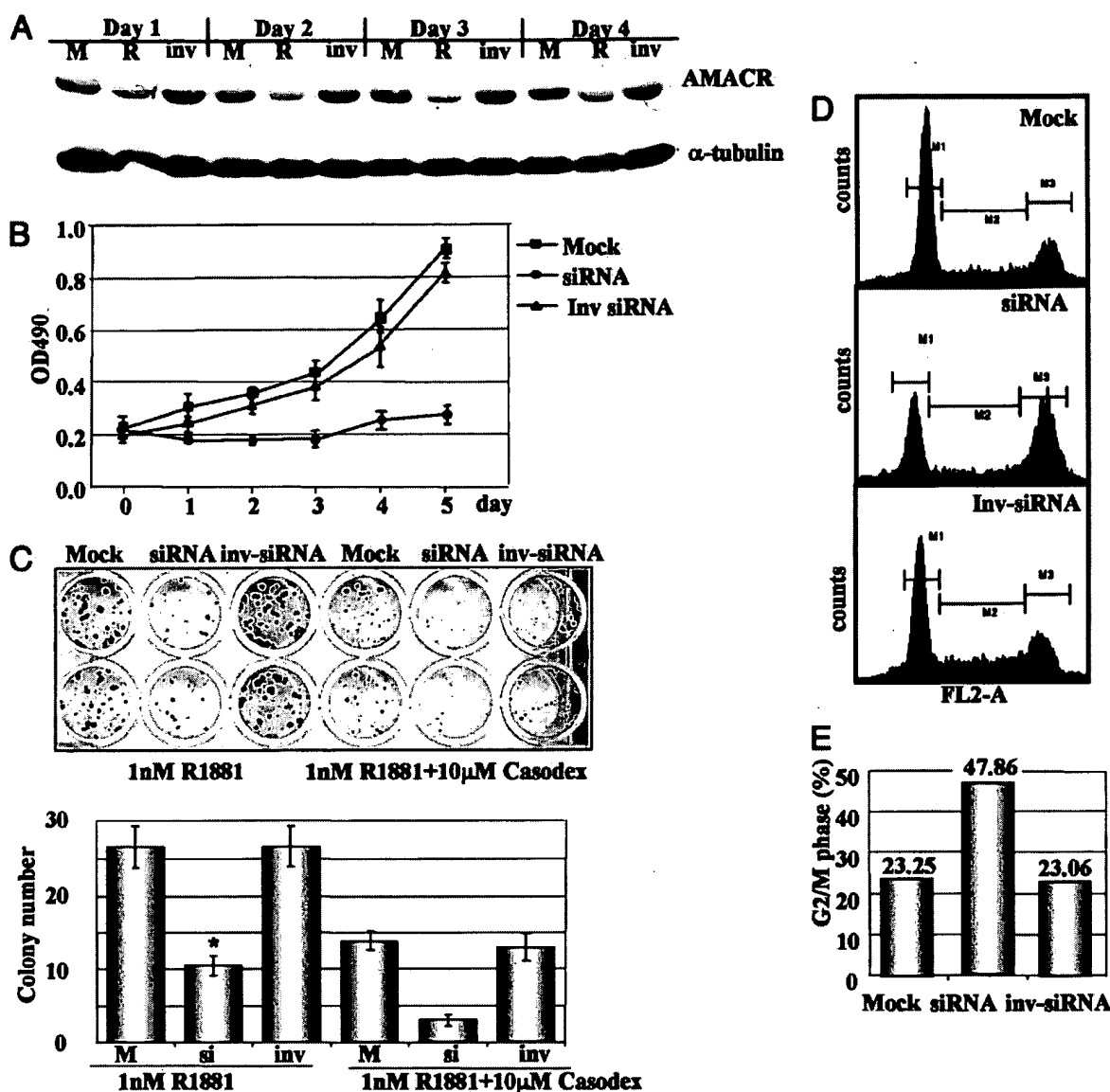


Fig. 3. Growth analyses of LAPC-4 cells after siRNA treatment. *A*, Western blot with 20 μg of total protein lysate from LAPC-4 cells during the course of growth analysis (start from 48 h after the second electroporation). *B*, proliferation of LAPC-4 cells was analyzed by mitochondrial activity-based assay. The results shown are representative of triplicate experiments. *C*, crystal violet staining of low-density LAPC-4 cells (250 cells/well) cultured for 14 days after the second electroporation with or without 1 nM R1881 or with 1 nM R1881 + 10 μM Casodex. The results are representative of quadruplicate experiments. The bar graph represents the number of colonies counted from one of three experiments. \*,  $P < 0.01$  when compared with mock ( $t$  statistics = 8.41) or invRNA ( $t$  statistics = 10.15) by paired Student's  $t$  test. *D*, DNA content-based cell cycle analysis of the same siRNA-treated cells 48 h after the second treatment. *E*, bar graph summarizes the percentage of cells in G<sub>2</sub>-M phase of cell cycle under different treatment conditions.

protein induced on the androgen deprivation required for the assay. In both cases, CWR22-Rv1 and LAPC-4 exhibited AR activity on treatment with synthetic androgen R1881 (Fig. 6, D–G). Loss of AMACR expression by siRNA treatment did not affect the basal level or the induced level of the AR reporter (Fig. 6, D and E). Furthermore, transient expression of wild-type or mutated (L107P loss of function mutation) AMACR did not change AR activation (Fig. 6, F and G). Together, these data suggest that the AMACR is not essential and not sufficient to drive AR-targeted gene expression in PCA.

**AMACR Is Not an AR-Targeted Gene in PCA.** In addition to an effect of AMACR on AR signaling, it was important to examine whether the expression of AMACR was regulated by AR. AMACR expression in three AR-positive PCA cell lines was measured after androgen stimulation (CWR22-Rv1 and LNCaP) or withdrawal (LAPC-4) over a 5-day course. In all three cell lines, higher steady-state AR level is associated with the presence of androgen (Fig. 7,

A–C). In LNCaP cells, the expression level of AR target gene PSA increased in a biphasic mode as described in the literature (Ref. 26; Fig. 7C). However, no detectable change in AMACR protein level was observed in any of these three cell lines (Fig. 7, A–C). Consistent with previously published results, these data indicate a hormone-independent regulation of AMACR in these cell lines (29). To directly determine the impact of AR signaling on AMACR transactivation, a 5000-bp region immediately upstream of the human AMACR gene was scanned for androgen response elements using Patch 1.1 on the Transfact website (30).<sup>4</sup> No binding site more than 65% identical to the consensus sequence (AGAACANNNTGTTCT) was found. Based on these results, we inferred that if AR signaling had any effect on AMACR expression, it would be subtle. A more sensitive, luciferase-

<sup>4</sup> <http://www.gene-regulation.com/>.

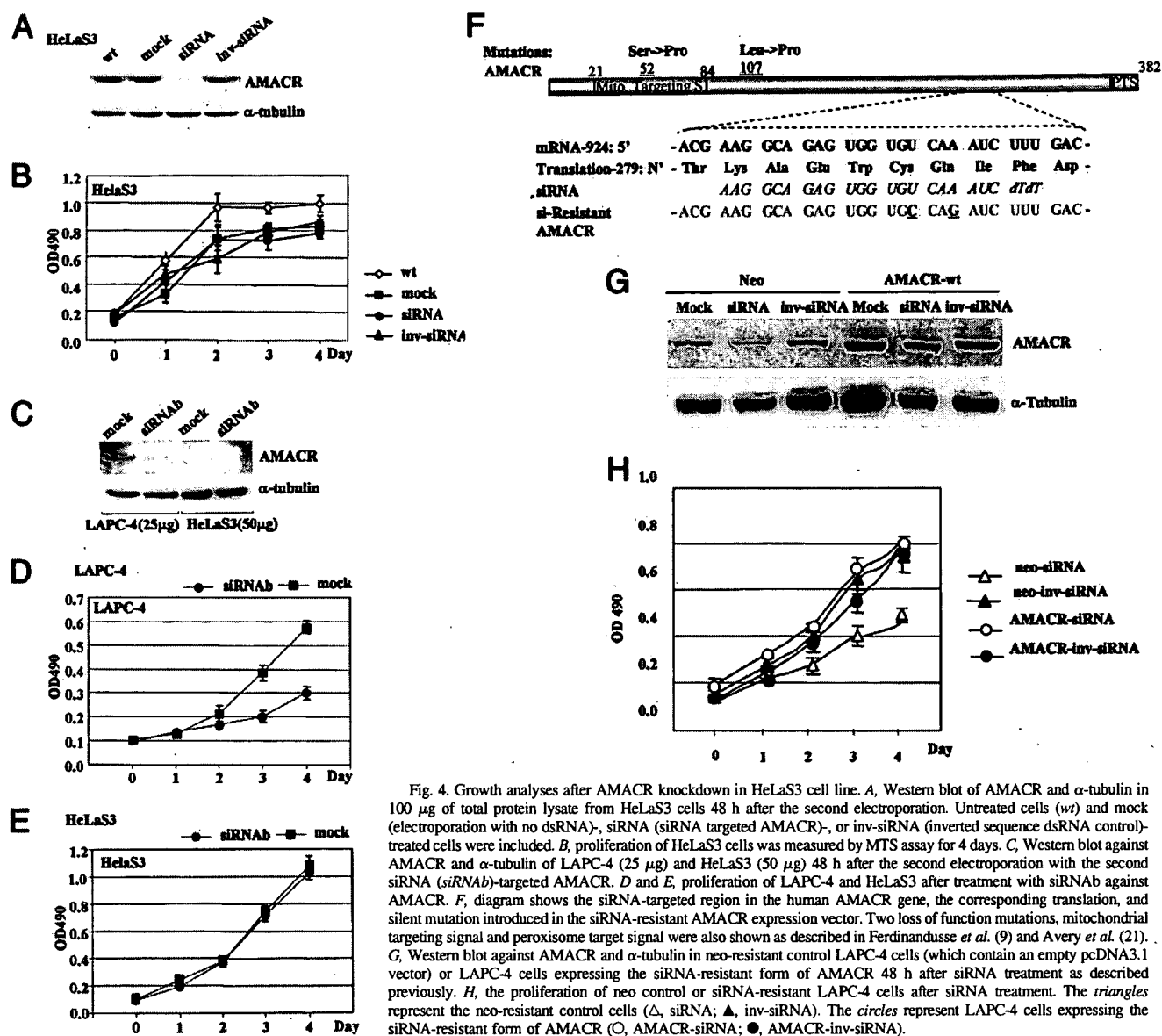


Fig. 4. Growth analyses after AMACR knockdown in HeLaS3 cell line. **A**, Western blot of AMACR and α-tubulin in 100 μg of total protein lysate from HeLaS3 cells 48 h after the second electroporation. Untreated cells (wt) and mock (electroporation with no dsRNA)-, siRNA (siRNA targeted AMACR)-, or inv-siRNA (inverted sequence dsRNA control)-treated cells were included. **B**, proliferation of HeLaS3 cells was measured by MTS assay for 4 days. **C**, Western blot against AMACR and α-tubulin of LAPC-4 (25 μg) and HeLaS3 (50 μg) 48 h after the second electroporation with the second siRNA (siRNA)-targeted AMACR. **D** and **E**, proliferation of LAPC-4 and HeLaS3 after treatment with siRNA against AMACR. **F**, diagram shows the siRNA-targeted region in the human AMACR gene, the corresponding translation, and silent mutation introduced in the siRNA-resistant AMACR expression vector. Two loss of function mutations, mitochondrial targeting signal and peroxisome target signal were also shown as described in Ferdinandusse *et al.* (9) and Avery *et al.* (21). **G**, Western blot against AMACR and α-tubulin in neo-resistant control LAPC-4 cells (which contain an empty pcDNA3.1 vector) or LAPC-4 cells expressing the siRNA-resistant form of AMACR 48 h after siRNA treatment as described previously. **H**, the proliferation of neo control or siRNA-resistant LAPC-4 cells after siRNA treatment. The triangles represent the neo-resistant control cells (Δ, siRNA; ▲, inv-siRNA). The circles represent LAPC-4 cells expressing the siRNA-resistant form of AMACR (○, AMACR-siRNA; ●, AMACR-inv-siRNA).

based reporter assay was therefore used to study the influence of AR signaling on AMACR expression. We cloned the promoter/enhancer and the complete 5'-UTR (5'UTR, -1711 to +87) of human AMACR and inserted it into pGL3<sub>Basic</sub> to form a seamless joint (no additional sequences from the vector were present between the 5'-UTR from AMACR and luciferase gene in the reporter) with the luciferase gene (pGL3<sub>Basic</sub>-AMACR). The high basal activity of this reporter in CWR22-Rv1 and LAPC-4 cells implied that the cloned sequence is sufficient to support promoter activity (Fig. 7, *E* and *G*). Heterologous AR reporter pBK-PSE-PB was used as an indicator for AR activation. Both CWR22-Rv1 and LAPC-4 exhibited AR activity on treatment with synthetic androgen R1881, as indicated by ~8-fold increase of pBK-PSE-PB-driven luciferase activity (Fig. 7, *D* and *F*). Meanwhile, no significant change on the pGL3<sub>Basic</sub>-AMACR activity was observed (Fig. 7, *E* and *G*). Increased ectopic expression of AR enhanced the activation of pBK-PSE-PB, but not the pGL3<sub>Basic</sub>-AMACR (Fig. 7, *D-G*). Together, these data strongly suggest that the expression of both AMACR protein and mRNA are not regulated by AR in PCa cells.

## DISCUSSION

In summary, our study reveals for the first time that the previously reported increases of AMACR at the protein and mRNA levels in clinical specimens of PCa are accompanied by increased enzymatic activity as well. We show that this increase is not associated with general peroxisome/mitochondrion proliferation. Furthermore, using the PCa cell line LAPC-4 as an *in vitro* model, we demonstrate that knockdown of AMACR expression in PCa cells impairs their proliferation. The mechanism of this growth inhibition is independent of androgen and, in fact, can be combined with androgen ablation to achieve an additive growth-inhibitory effect.

Previous studies have suggested multiple pathways through which metabolic enzymes might be involved in the growth regulation of cancer cells, *e.g.*, maintaining essential energy equilibrium, preserving reduction/oxidation balance, and generating/degrading critical signaling molecules (31). Increased glycolysis and fatty acid synthesis were described in various cancer types including PCa (31). In addition, tumorigenesis in the prostate is also associated with loss of normal

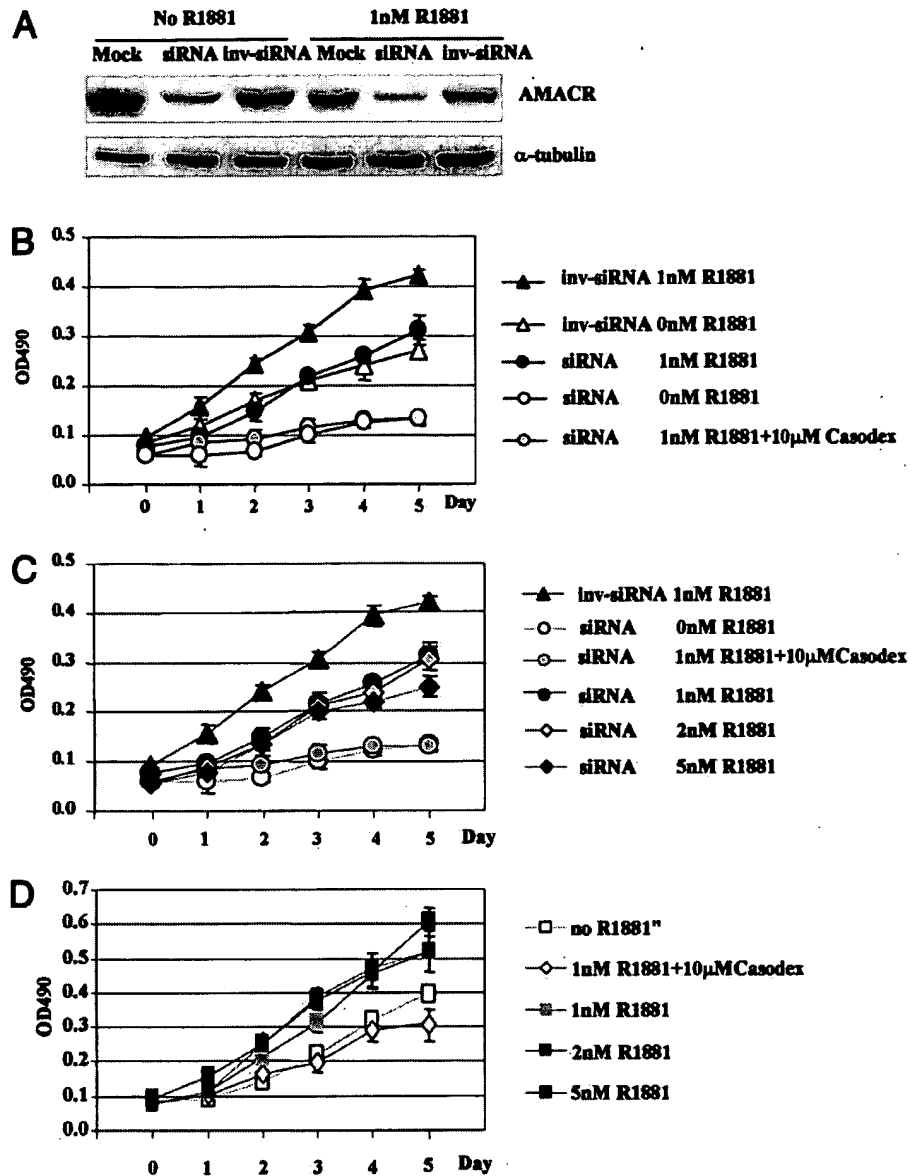


Fig. 5. Proliferation analysis of LAPC-4 cells treated with siRNA and androgen ablation. **A**, Western blot against AMACR and  $\alpha$ -tubulin of 20  $\mu$ g of total protein from LAPC-4 cells 48 h after the second electroporation. **B**, proliferation of LAPC-4 cells treated with either inv-siRNA or siRNA and then cultured in 0 nM R1881, 1 nM R1881 or 1 nM R1881 + 10  $\mu$ M Casodex. **C**, proliferation of LAPC-4 cells treated with siRNA and cultured in different concentrations of R1881. **D**, proliferation of untreated LAPC-4 cells cultured in different concentrations of R1881 or R1881 + Casodex. Notice the scale in this graph is larger than the one in **B** and **C**. **E**, crystal violet staining of low-density LAPC-4 cells (250 cells/well) cultured for 20 days with different concentrations of R1881 after siRNA treatment or control treatments. Two wells for each condition are shown, representative of quadruplicate experiments. **F**, cell cycle analysis of siRNA-treated cells after incubation in medium containing different concentrations of R1881 as indicated for 48 h.

prostate-specific metabolic features such as the ability to concentrate zinc in mitochondria and the secretion of citrate. It is generally believed that these metabolic changes do not cause cancer but may be required to satisfy the increased energy demands accompanying transformation (32). Correspondingly, specific inhibition of these pathways might provide new therapeutic targets for treatment of PCa. As an example, fatty acid synthase inhibitors cerulenin and C75 have potent activities as selective inhibitors of tumor cell growth (33). It is possible that elevated expression of AMACR and the concomitant increase in enzyme activity may enable PCa cells to use new energy sources or generate novel metabolites (e.g., cholesterol derivatives) with possible growth-promoting activities.

Peroxisomal  $\beta$ -oxidation consists of two separate pathways. One is the classical peroxisome proliferator-inducible pathway responsible for the degradation of most straight-chain acyl-CoAs through fatty acyl-CoA oxidase, L-bifunctional protein, and thiolase. The other one is the noninducible pathway catalyzing the oxidation of 2-methyl-branched fatty acyl-CoAs, by branched-chain acyl-CoA oxidase, D-bifunctional protein, and sterol carrier protein X (34), with the exception that D-bifunctional protein is also required for  $\beta$ -oxidation of

very-long-chain fatty acid (35). In the latter case, AMACR is required for the complete degradation of branched-chain fatty acids and the synthesis of bile acids. Curiously, neither of these functions has been implicated previously as playing important roles in the biology or physiology of normal prostate or PCa. Also, it is not clear whether AMACR is the rate-limiting step of this second pathway, in general, or in the prostate, in particular. We are currently assessing the possible activation or overexpression of other members in the branched-chain fatty acid  $\beta$ -oxidation pathway to see whether the complete pathway is induced. Previous studies provide some interesting information on the general role of fatty acid oxidation in PCa; however, no specific inference was drawn regarding branched-chain fatty acid oxidation in particular. Studies with fatty acid synthesis inhibitors suggested that inhibition of fatty acid synthesis by itself had no effect on growth; rather, simultaneous inhibition of both fatty acid synthesis and  $\beta$ -oxidation was required for the cancer-specific cytotoxic activity of the fatty acid synthase inhibitors cerulenin and C75 (36). Whereas these studies emphasize the special role that  $\beta$ -oxidation may play in PCa in general, how much of this effect is due to modulation of branched-chain fatty acid  $\beta$ -oxidation in the peroxisome, if any, is not known.

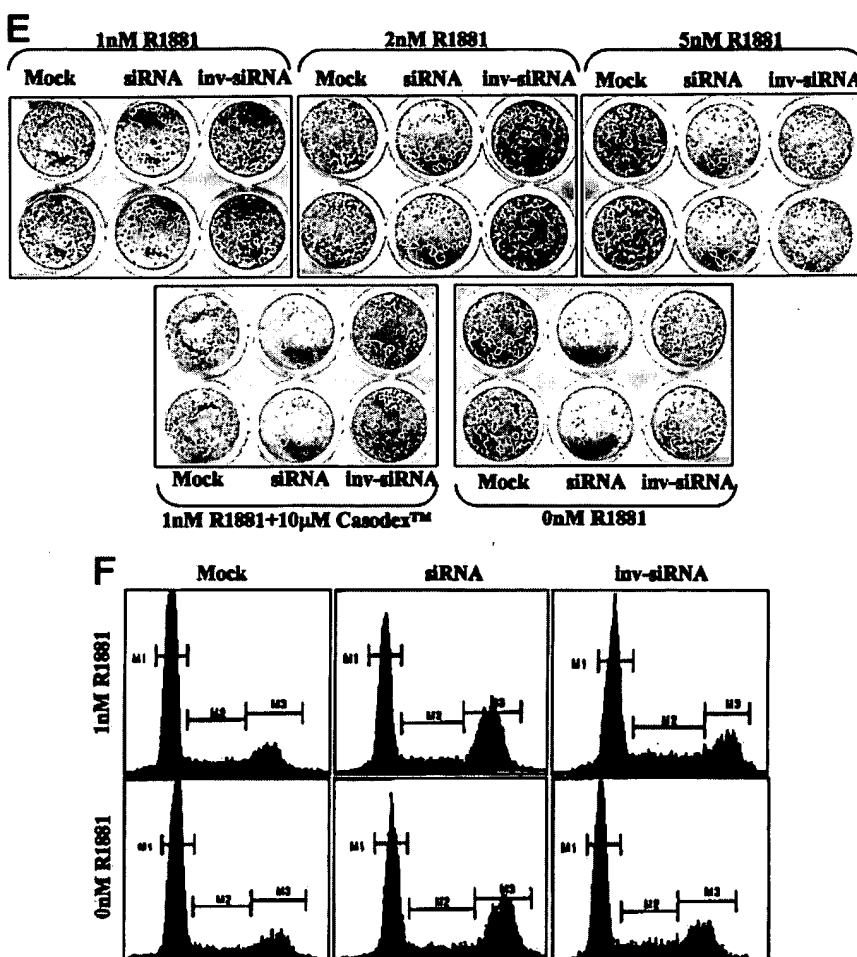


Figure 5. Continued.

Nonetheless, the consistently enhanced activity of AMACR in PCa opens the possibility for a role of branched-chain fatty acid  $\beta$ -oxidation in tumorigenesis and thus the pathway as a potential therapeutic target.

$\beta$ -oxidation of branched-chain fatty acids such as pristanic acid might provide a critical energy source in the course of tumorigenesis in prostate, and inhibiting this activity might slow growth. Another possibility is that inhibiting the oxidation of pristanic acid leads to an increased concentration of this fatty acid, with a resultant associated toxicity, especially because its accumulation in the neuronal system is thought to be the cause of neuropathy in patients with genetic deficiencies of AMACR. However, it is unlikely that the growth inhibition we observed here after siRNA knockdown is due to differences in the sensitivity of the cell lines to this fatty acid. In contrast to adult cow serum, FBS used in all of the culture media in this study is virtually devoid of phytanic acid and pristanic acid (37). The average pristanic acid concentration in human plasma ranges from 0 to 2  $\mu$ M and can reach over 100  $\mu$ M in patients with hereditary AMACR mutations. Furthermore, when SCP<sub>2X</sub><sup>-/-</sup> mice, which are incapable of metabolizing branched-chain fatty acids due to an inability to catalyze the thiolitic cleavage of 3-keto-pristanoyl-CoA, were fed a phytol-containing diet, the plasma concentration of phytanic acid, the precursor of pristanic acid, was >1 mM. Even at this condition, phytanic acid did not cause life-threatening symptoms. In general, the development and reproduction of the SCP<sub>2X</sub><sup>-/-</sup> mouse is normal (38).

Other than containing an  $\alpha$ -methylacyl-CoA thioester, the consen-

sus structure of AMACR substrates has not yet been identified. This opens the possibility for a broad spectrum of chemical structures to act as substrates. In fact, the first function identified for AMACR is the chiral inversion of 2-arylpropionic acid nonsteroidal anti-inflammatory drugs (e.g., ibuprofen and flurbiprofen; Ref. 39, 40). Thus, it is possible that AMACR in PCa acts on some unidentified substrates, which might be involved in growth control.

Unlike mitochondrial  $\beta$ -oxidation, peroxisomal  $\beta$ -oxidation generates H<sub>2</sub>O<sub>2</sub>, which is degraded by catalase in the peroxisome. The finding that no up-regulation of catalase is observed concomitantly with extensive up-regulation of AMACR may suggest a shift in the balance between production and degradation of reactive oxygen species, possibly favoring a pro-oxidant environment. Previous studies have established that PCa has a reduced ability to deal with oxidative stress due to loss of expression of glutathione *S*-transferase  $\pi$  through promoter hypermethylation early in disease progression (41, 42). Under this condition, increased activity of the peroxisomal  $\beta$ -oxidation pathway might accelerate oxidative damage and potentially promote genetic instability. Such a mechanism has been proposed to explain the ability of ectopic overexpression of peroxisome acyl-CoA oxidases to transform tissue culture cells (43). However, the role of constitutively active peroxisomal  $\beta$ -oxidation in established cancer is still largely an open question.

It is also worth noting that increased, cancer-associated AMACR activity in the prostate offers a possible selective drug delivery route. For instance, when ibuprofen is given as a racemic mixture (which it typically is), PCa cells would have a higher concentration of the active

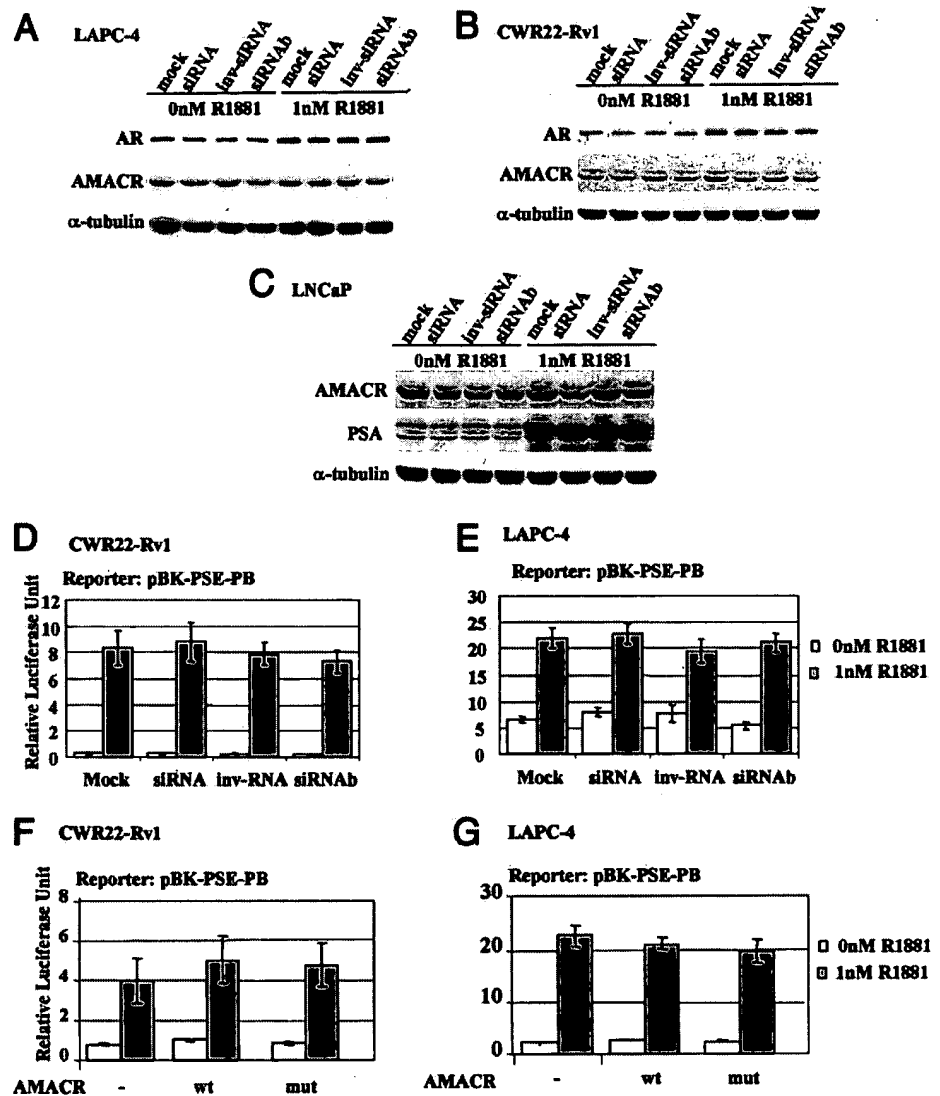


Fig. 6. AMACR alone is neither essential nor sufficient to drive AR-targeted gene expression. A–C, Western blots show the expression of AR, AMACR, PSA, and  $\alpha$ -tubulin in LAPC-4 (A), CWR22-Rv1 (B), and LNCaP (C) after single siRNA treatment and 48 h of incubation in medium with or without 1 nM R1881. D and E, the activities of pRK-PSE-PB-luc were not affected by siRNA-targeted disruption of AMACR in CWR22-Rv1 (D) and LAPC-4 (E). F and G, AR-induced pRK-PSE-PB-luc activity was not affected by ectopic expression of wild-type AMACR or mutated AMACR (L107P) in CWR22-Rv1 (F) and LAPC-4 (G).

form (in terms of cyclooxygenase inhibition) than normal prostate epithelial cells, due to the increased ability of the cancer cells to convert (*R*)-ibuprofen (inactive form) to (*S*)-ibuprofen (active form) through AMACR. The specific enzymatic function of AMACR suggests small chemical inhibitors as a more attractive method to abolish its functions than a siRNA-mediated approach. However, it should be kept in mind that the existence of other nonenzymatic functions of AMACR and their possible contributions to the growth regulation in prostate cannot be ruled out at this moment. Lack of homology to other proteins with known structure makes it difficult to generate specific active site mutation(s) and directly test whether inhibition of AMACR activity is equivalent to knocking down the protein with siRNA. During the course of this study, we did generate and test two AMACR expression constructs carrying loss of function mutations (S52P and L107P) characterized by Ferdinandusse *et al.* (10) in patients with genetic deficiency of AMACR. Ectopic expression of these mutants was not able to restore normal proliferation after siRNA treatment (data not shown). However, the structural impact of these mutations (both to proline) to AMACR is not clear. They may potentially destroy the overall folding of AMACR and abolish whatever function(s) that AMACR might have along with the enzymatic activity. A recent publication described the successful crystallization and preliminary X-ray diffraction of AMACR from *Mycobacterium*

*tuberculosis* (44). Solving of this structure would assist in understanding the structure and function of human AMACR based on the high homology on protein sequence. Further structural analysis is necessary to provide a solid validation for small chemical inhibitor strategy.

As part of the characterization of AMACR in PCa, we addressed the regulation by and interaction between AMACR and AR. Previous immunohistochemistry studies of AMACR in different stages/grades of PCa have yielded different conclusions regarding this issue. One set of studies from three different groups agreed that the intensity of AMACR staining remains unchanged, whereas PCa progresses to high grades or stages (3, 6, 7); on the other hand, Kuefer *et al.* (29) indicated that hormone-refractory cancers expressed AMACR to a lesser extent and that cellular differentiation levels inversely correlated with AMACR staining. Furthermore, in our study and in studies from other groups, the expression and activity of AMACR in PCa cells are high in AR-positive cell lines (LNCaP, LAPC-4, and CWR22-Rv1) but low in AR-negative cell lines (DU145 and PC-3; Ref. 29). Here we provide evidence for an androgen-independent role of AMACR in the growth regulation of PCa. AMACR could not affect the stabilization of AR itself or modulate the expression of the AR-targeted gene PSA *in vivo* or in the reporter assays. Meanwhile, the expression of AMACR itself is not regulated by AR. It is interesting to note that male sterol carrier protein X-null mice, which are



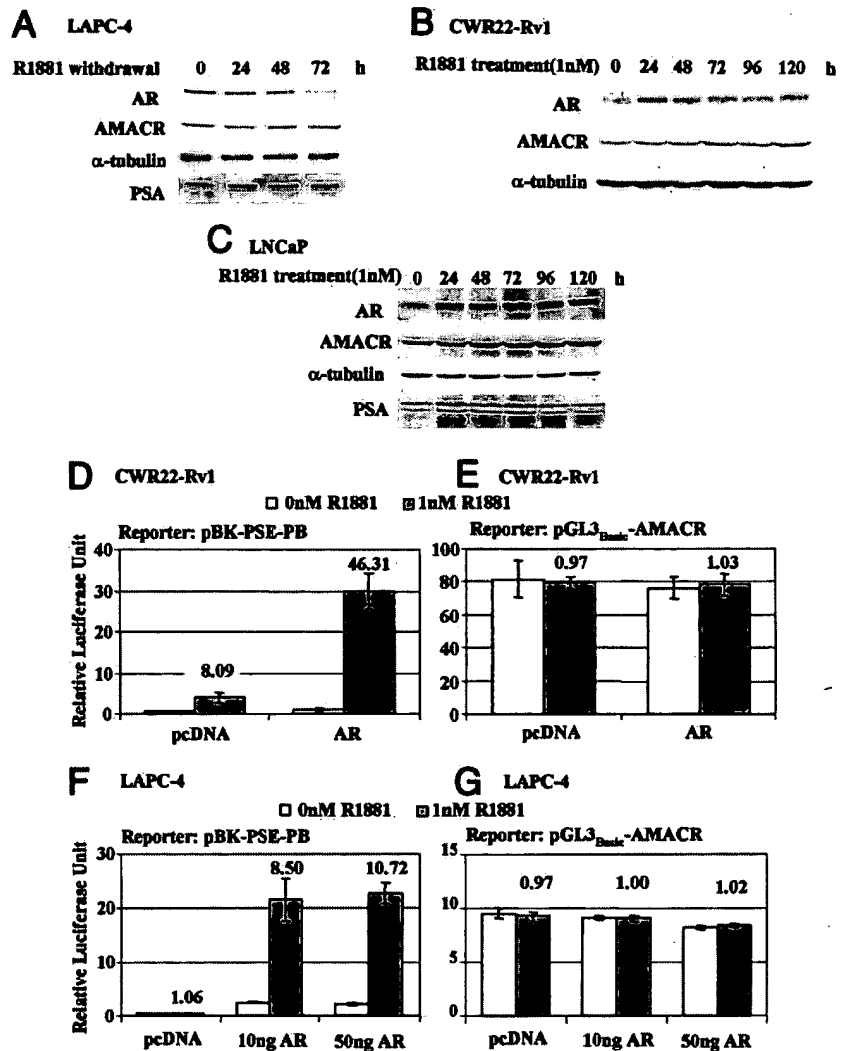


Fig. 7. The expression of AMACR was not changed on AR activation. A–C, Western blots show the expression of AR, AMACR, PSA, and  $\alpha$ -tubulin in LAPC-4 (A), CWR22-Rv1 (B), and LNCaP (C) cells treated with 1 nM R1881 for the indicated times after 2 days of androgen deprivation (for CWR22R or LNCaP) or withdrawal of R1881 (from 1 nM to 0 nM R1881, in the case of LAPC-4) for different lengths of time. D–G, the relative luciferase activities derived from pBK-PSE-PB (D and F) or pGL3<sub>Basic</sub>-AMACR (E and G) after stimulation with 1 nM R1881 for 24 h in CWR22-Rv1 (D and E) and LAPC-4 (F and G). The amount of ectopic AR introduced into the transfection mixture was labeled on the bottom of each graph. The numbers above each shaded column represent the average fold of induction by 1 nM R1881.

defective for branch-chained fatty acid  $\beta$ -oxidation, have normal testosterone levels and reproductive abilities, suggesting that this pathway is not generally related to AR signaling. However, caution needs to be exercised when expression of prostate differentiation markers (e.g., PSA, prostate-specific membrane antigen, or human kallikrein 2) are used as surrogates for the growth-modifying function of AR because they are frequently dissociated from each other, as emphasized in a recent study (25). The disassociation maybe related to the presence of gene-specific coactivators for AR or complicated cross-talk between AR and other signaling pathways. In agreement with this, AR regulates the growth but not the expression of PSA in our analysis of LAPC-4 cells *in vitro*. The androgen-independent feature of AMACR could be beneficial as a therapeutic approach, especially in the case of hormone-refractory PCa. However, more work is needed to demonstrate AR independence *in vivo*, especially under clinical conditions of androgen ablation, when alterations in AR signaling mechanisms are frequently seen, and interactions between different cell types are important.

Finally, a shift from paracrine to autocrine in terms of androgen supply has been implicated in PCa (45). Given the role that AMACR plays in bile acid production and the structural similarity between cholesterol metabolites and sterols, it is possible that AMACR contributes to this shift by increasing the level of as yet undefined androgenic factors. The fact that we did not see any effect on AR

signaling by expressing AMACR would argue against this, however. It is still possible that this lack of an androgenic effect could be due to the unavailability of other enzymes or coactivators in the *in vitro* cell line system; therefore, the existence of possible interactions *in vivo* cannot be ruled out. Hopefully, transgenic models will soon become available to better understand the function of AMACR in PCa development and to address the endocrinological details of AMACR in PCa.

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